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Detection of the Resistance of Parasite to Sulfadoxine-pyrimethamine Drugs and msp-2 Genotyping as A Baseline in Developing Malaria Vaccine with Ionizing Radiation

Deteksi Resistensi Parasit terhadap Obat Sulfadoksin-Pirimetamin dan Genotyping msp-2 sebagai Dasar Pengembangan Vaksin Malaria dengan Radiasi Pengion

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ABSTRAK

Polimorfisme atau mutasi pada gen spesifik *P. falciparum* dan *P. vivax* terlibat dalam resistensi terhadap obat antimalaria sulfadoksin-pirimetamin (SP). Di sisi lain gen *msp-2* berperan penting dalam genotipe terkait resistensi obat. Studi ini dilakukan sebagai informasi dasar dalam menilai urgensi pengembangan vaksin malaria yang dapat dibuat dengan radiasi pengion. Asam deoksiribonukleat (DNA) darah dari pasien rawat jalan yang terinfeksi malaria di rumah sakit Dok II Jayapura pada periode Nopember 2014 diamplifikasi dengan menggunakan *nested polymerase chain reaction* (PCR) dan diikuti oleh analisis *restriction fragment length polymorphism* (RFLP) untuk menentukan polimorfisme resistensi SP. Di antara 15 sampel yang diuji untuk gen *dhfr*, 9 (60%) dan 8 (53%) sampel menunjukkan hasil positif untuk polimorfisme berturut-turut dengan primer JR78/79 dan F/108DH. Untuk gen *dhps* dengan menggunakan primer JR84/85 dan L/L, 7 sampel positif mutan. Frekuensi ini lebih rendah dibandingkan dengan hasil penelitian lain. Dari 15 sampel yang diperiksa, 3 memiliki alel 3D7 dan 4 memiliki alel FC27 dari gen *msp2*. Tidak ada alel S1105 dan S1240 bermutasi dan 6 alel VDT mutan untuk *P. vivax*. Dapat disimpulkan bahwa resistensi parasit terhadap SP cukup tinggi, hal ini menunjukkan sangat pentingnya mengembangkan vaksin malaria.

Kata kunci : resistensi, dhfr, dhps, mutasi, vaksin malaria

ABSTRACT

Polymorphism or mutation in specific genes of *P. falciparum* and *P. vivax* is involved in the resistance to sulfadoxine-pyrimethamine (SP) antimalarial drug. On the other hand *msp-2* gene plays an important role in drug resistance related genotyping. This study was undertaken as a basic information in assessing the urgent of development of malaria vaccine that can be created by ionizing radiation. Deoxy ribonucleic acid (DNA) of blood from malaria infected outpatients in Dok II Hospital of Jayapura for November 2014 period was amplified using nested polymerase chain reaction (PCR) and followed by restriction fragment length polymorphism (RFLP) analysis to determine the polymorphisms of SP resistance. Among 15 samples tested for *dhfr* gene, 9 (60%) and 8 (53%) samples showed positive result for polymorphisms in JR78/79 and F/108DH primers, respectively. For *dhps* gene by using JR84/85 and L/L primers 7 samples were positive mutant. These frequencies are lower compared to results of other research. of these 15 samples examined, 3 had 3D7 alleles and 4 had FC27 alleles of the *msp2* gene. No mutated S1105 and S1240 alleles and 6 mutant VDT alleles were found in *P. vivax*. It can be concluded that the resistance of parasites to SP was quite high, indicating the highly urgency to develop malaria vaccine.

Keywords : *resistance, dhfr, dhps, mutation, malaria vaccine*

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INTRODUCTION

Malaria is one of the diseases with a high prevalence rate in the world. Approximately 3.3 billion people in 97 countries and territories are at risk of being infected with malaria. According to the World Health Organization (WHO), morbidity and mortality due to malaria tend to decline in the period 2005-2015. In 2015, an estimated 214 million new cases of malaria occurred with the death of around 438 thousand people worldwide [1]. Indonesia with a population of around 265 million is one country that has a high mortality rate in malaria cases. This country is also a place for more than 20 *Anopheles* vectors of malaria which transmit the four species of *Plasmodium sp*. which infects humans [2]. By implementing all efforts Indonesia marked a milestone in 2017 with more than half of districts officially declared malaria free.

Malaria is a disease that originates from *Plasmodium sp*. infection and including deadly diseases both in animals and humans [1], [3]. *P. falciparum* and *P.vivax* are parasites that cause the greatest health problems in Indonesia, where most of the deaths are caused by these two parasites. A total of 56% of *P. falciparum* parasites and 44% of *P. vivax* parasites were recorded as the two species that infected malaria in Indonesia in 2013 [4]. The highest prevalence of malaria caused by *P. falciparum* parasites is found in the eastern part of Indonesia, especially in the Papua region [2].

SP or Fansidar antimalarial drugs are used as a second-line treatment by inhibiting the action of the dihydrofolate reductase (*dhfr*) and dihydropteroate synthase (*dhps*) genes in folate biosynthetic pathways, and are believed to increase antimalarial potential and reduce the risk of drug resistance. However, resistance to this drug combination was reported to occur shortly after the introduction of the drug. The spread of resistance to SP drugs has become a public health problem because the drug is still used as an alternative to malaria treatment [5]. Beside that genetic diversity of the gene coding for merozoite surface protein 2 (*msp2*) of *P. falciparum* that is abundant surface protein on the blood stage of parasite can be used in understanding malaria control and elimination programs. The locus of this gene is extremely polymorphic with two allelic families (3D7 and FC27), both of which are closely associated with clinical manifestation, severity of malaria and demographic factors [6], [7].

Plasmodium sp. parasite resistance against antimalarial drugs can be caused by many factors. Molecular studies that carried out by detecting the mutations of *dhfr and dhps* genes are believed to have an effect on antimalarial drug resistance [8]. Further molecular studies need to be done by looking at the polymorphism to determine the effect of genes on antimalarial drug resistance. Genetic variation in *P. falciparum* is reported to be quite high and results in new parasites. This gene variability can also be used to see the frequency of recurrent infections in humans [9]. Dihydrofolate reductase (*dhfr*) is a gene that influences folic acid biosynthesis [10] and is targeted by pyrimethamine drugs in malaria parasites. Mutations in this gene are related to resistance to pyrimethamine drugs [11]. Resistance to other antimalarial drugs of sulfadoxine is related to mutations in the *dhps* gene. Detection of mutations in both genes is widely done to see the correlation between mutations with drug resistance. On the other hand, the *pvdhfr* and *pvdhps* genotypes might also be associated with treatment failure in individual vivax malaria-infected patients [12].

Analysis of genetic variation that has accurate results has a lot of influence on biomedical research. Analysis with method such as an RFLP is widely used to detect the presence of single nucleotide polymorphisms (SNP) [13]. One other alternative to detect mutation is single-strand conformation polymorphism (SSCP) with high accuracy and sensitivity [14]. More recently it is also described protocols that utilize silver staining or with fluorescent dyes [15].

The existing frontline malaria control interventions such as vector control by indoor residual spraying are expensive and even not effective due to the emergence of drug resistance [16]. It calls for an innovative approach in terms of potential and reliable vaccine as an additional tool and could be primed for breakout. Ionizing radiation has become a reliable tool to create a cost-effective vaccine for this deadly disease, where early our study found that 150 Gy of

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gamma rays was effectively to attenuate parasites as vaccine material [17], [18].

This study aims to detect mutations in the genes encoding antimalarial drug resistance and genetic variation in Plasmodium, namely the *dhfr and dhps* genes in *P. falciparum* and *P. vivax* parasites as well as *msp-2* with PCR-RFLP as a basic information for the development of malaria vaccine in Indonesia to achieve effective disease control.

MATERIALS and METHODS

Object of Research

The object of this study was DNA sample of *P. falciparum* and *P. vivax* parasites which had been extracted from blood samples dropped on a filter paper of malaria infected patients (with no previous information on its drug resistance) attending the Dok II Hospital, Jayapura City, Papua Province. The blood sampling was done in September 2014. Monitoring of parasite infection in the blood samples was performed using microscopic evaluation of thick blood smear that was first fixed with methanol (Merck), and then stained with Giemsa (Sigma). Fifteen samples obtained from 9 males and 6 females (due to limited time of sampling) that were stored individually in zip-lock bags were tested for *dhfr and dhps* polymorphisms.

Ethical of the Research

Research procedures and obtaining blood samples of suspected of malaria patients have been approved by the Ethics Commission, National Institute of Health Research and Development, Ministry of Health in Jakarta (No. LB.02.01/5.2/KE.162/2014 dated of 24 April 2014, and permitted from Jayapura General Hospital, Papua, Indonesia. Informed consent was given to all respondents or patients being infected with malaria who participated in this research.

Microscopic observation

Malaria species infecting the blood samples was determined by the thick smears. A drop of blood obtained from patient was put on the clean glass slide, air dried and then was stained with Giemsa following standard procedures.

DNA extraction

The DNA of *P. falciparum* parasite was extracted from blood sample using Chelex-100 ion exchanger (Biorad Laboratories, Hercules, CA, USA) and followed the standard procedures as described by Asih *dkk.* [19]. The filter paper with dropped blood was placed in a microtube, 100 µl distilled water and 50 µl 20% Chelex-100 in distilled water were added. DNA was extracted by boiling it at 100 °C for 10 min. DNA samples were then be used immediately for PCR amplification or stored in a freezer at -20°C for later analysis.

Amplification of DNA with nested PCR

PCR amplification for genotyping of SP drug resistance encoding *dhfr* and *dhps* genes was carried out using the nested PCR principle. PCR was carried out at 25 µL reaction volume containing 1x Buffer, 1.57 mM $MgCl_2$ (Invitrogen), 120 µM of dNTP, 0.4 units of Taq DNA polymerase, 30 µM of each oligonucleotide primer and 5 µL of DNA template. The nested PCR method is used to amplify the resistance encoding genes because this method can increase the sensitivity of gene mutation detection. In nested PCR, the amplification reaction was performed twice. In the first reaction, the oligonucleotide primer sandwiches the polymorphic region of the gene used. In the second reaction, the first product PCR reaction of 2 µL was used as a DNA template (amplicon) (Figure 1). The primary nucleotide sequences used in nested-PCR are shown in Table 1 where the pair of primers is as follow: JR84-JR85, L-L', JR78-JR79, F-108DH for *P. falciparum*, VDT of - VDT ON and VDT-OF – VDT-OR for *P. vivax*, and S2-S3 and S1-S4 for *msp-2* genotyping. The PCR conditions were performed according to Abdullah *dkk.* [20] and Imwong dkk. [21]. Briefly, the PCR (Applied Biosystems, USA) involved 30 amplification cycles composed of initial denaturation at 94°C for 5 minutes, a denaturing step at 94°C for 1 minute, followed by annealing at 55°C for 2 minutes, and an extension step for 2 minutes at 72°C.

For *msp-2* genotyping, in the initial amplification, primer pairs corresponding to conserved sequences within the polymorphic regions of gene were included in separate reactions. The product of initial amplification was used as a template in two separate nested PCR. In the nested reaction, separate primer pairs targeted the respective allelic types of *msp-2* (3D7 and FC27) with an amplification mixture containing 250 nM of each primer, 2 mM of $MgCl_2$ and

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125 µM of each dNTPs and 0.4 units Taq DNA polymerase. The cyclic conditions in the thermocycler for initial reaction were: 5 min at 95°C, followed by 30 cycles for 1 min at 94°C, 2 min at 58°C and 2 min at 72°C and final extension of 10 min at 72°C. For nested PCR, conditions were: 5 min at 95°C, followed by 30 cycles for 1 min at 95°C, 2 min at 61°C and 2 min at 72°C and final extension of 5 min at 72°C.

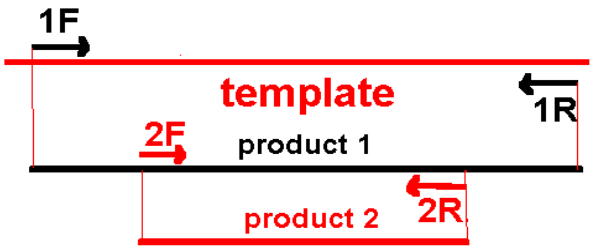

Figure 1. Schematic of nested PCR reactions. The arrow indicates the forward primer (1F) and reverse primer (1R) for the first round and the product 1 is then used as template for second round [22].

Detection of mutation with RFLP

The nested PCR products were then detected their mutation with restriction digestion. For *dhfr* and *dhps* of *P. falciparum*, the PCR products were digested with *Nla*III and *Tai*I (*dhfr*) and Msp A1I, Ava II (*dhps*) enzymes to determine the polymorphisms at codons of 15 and 60 (*dhfr*) and 436 and 437 (*dhps*) as described by Syafruddin *dkk.* [8] and Asih *dkk.* [19]. For *dhps* of *P. vivax*, the PCR products were digested with *Msp1* and *Msc1* enzymes as described by Asih *dkk.* [19] and Imwong *dkk.* [21] for the analysis of VDT allele. Digested products were subjected to electrophoresis on 1.5−3% agarose gels (Progen, Australia), stained with ethidium bromide, then observed under UV light, and photographed. A 100 pb ladder was used as a DNA size was used as a marker on all gels [19]. For *msp-2*, enzyme of *Hinf* I was used and members of the 3D7-type alleles produced two conserved restriction fragments (70 and 108 base pairs (bp)), whereas FC27-type alleles produced two conserved fragments (115 and 137 bp).

RESULTS and DISCUSSION

Detection of species

Based on microscopic observation it was known that from 15 samples analyzed, 9 samples were infected with *P. falciparum* (Figure 2) which characterized by the presence of young trophozoite and sausage-shaped gametocyte. Six were infected with *P. vivax* (Figure 2) which

Table 1. Sequence of nucleotide alleles / primers used in nested PCR for *dhfr*, *dhps* and *msp-2* genes either for the first round or second round of PCR [8], [19], [20], [21]

No.	Allele/ primer	Nucleotide array	References
dhfr – dhps			
P.falciparum			
1.	JR84	5'-GGT ATT TTT GTT GAA CCT AAA CG-3'	19-21
2.	JR85	5'-ATC CAA TTG TGT GAT TTG TCC AC-3'	
3.	L	5'-ATA GGA TAC TAT TTG ATA TTG GAC CAG GAT TCG-3'	19-21
4.	L'	5'-TAT TAC AAC ATT TTG ATC ATT CGC GCA ACC GG-3'	
5.	JR78	5'-CTC CTT TTT ATG ATG GAA CAA GTC-3'	19-21
6.	JR79	5'-CAT CAC ATT CAT ATG TAC TAT TTA TTC TAG T-3'	
7.	F	5'-GAA ATG TAA TTC CCT AGA TAT GGA ATA TT-3'	19-21
8.	108DH	5'-GGT TCT AGA CAA TAT AAC ATT TAT CC-3'	
P. vivax			
9.	VDT OF	5′-ATG GAG GAC CTT TCA GAT GTA TTT GAC ATT-3′	21
10.	VDT ON	5′-GGC GGC CAT CTC CAT GGT TAT TTT ATC GTG-3′	
11.	VDT OR	5′-CTT GCT GTA AAC CAA AAA GTC CA-3′	
Msp-2			
12.	S2	5'-GAA GGT AAT TAA AAC ATT GTC-3'	8

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13.	S3	5'-GAG GGA TGT TGC TGC TCC ACA-3'
14.	S1	5'-GAG TAT AAG GAG AAG TAT G-3'
15.	S4	5'-CTA GAA CCA TGC ATA TGT CC-3'

characterized by the presence of trophozoite and schizont and Schüffner stippling. Two samples were infected by both *P. falciparum and P. vivax*. Two samples were not infected either by *P. falciparum* or *P. vivax*.

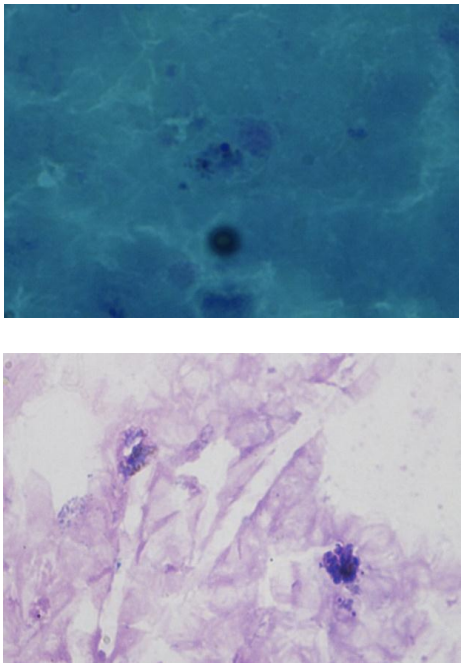

Figure 2. Microscopic observation of Giemsa stained thick smear of *P. falciparum* species (upper) of patient no. 1 and *P. vivax* species (below) of patient no. 4 infecting the blood samples with its own characteristics explained in text and the magnification of 1000X

Detection of mutated dhfr and dhps genes

DNA amplification with nested PCR is a very sensitive method for the detection of specific parasite species as its capable of identifying as few as 1-10 parasites/μL (low parasitemia) which may missed by microscopy observation, including mixed infections. In this study nested PCR amplification of DNA samples from *P. falciparum* infected patients followed by RFLP technique was done to detect mutations. The screening of the prevalence of these mutations could facilitate the surveillance of the level of S/P resistance *in vivo*. PCR amplification of two genes encoding anti-malaria drug resistance (*dhfr and dhps* genes) was carried out in 15 samples. The results showed that by using JR78/79 primer from *dhfr* gene, there were 9 samples which showed positive RFLP results, whereas with F/108DH primers there were 8 samples which showed positive RFLP results. The results of PCR amplification on *dhps* genes using JR84/85 primer and L/L primer showed that 7 samples were positive from 15 samples tested. For *P. vivax* polymorphism test there is no mutated S1105 and S1240 alleles and 6 mutant VDT alleles were found. All these results are presented in Table 2.

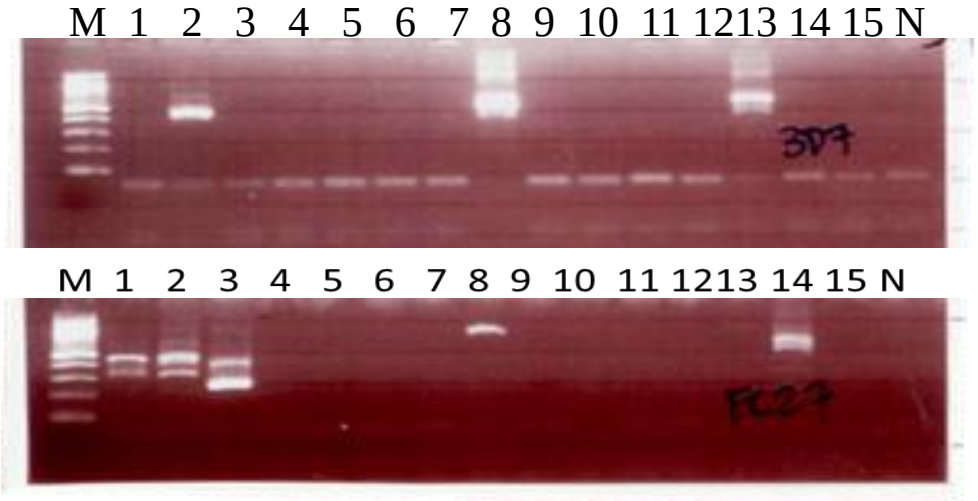

Figure 3. The PCR results for *msp-2* genotyping of *P. falciparum* with the appearances of 3D7 (upper) and FC27 (lower) alleles. M: marker of 100 bp, N: negative control

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Table 2. The results of RFLP analysis of *dhfr, dhps and msp-2* genes of *P. falciparum* and *P. vivax* parasites from blood samples of malaria infected Papua outpatients

No.	*Pfdhfr*		*Pfdhps*		*Msp-2*		*Pvdhfr*		*Pvdhps*
Sample	JR78/JR79	F/108DH	JR84/JR85	L/L"	3D7	FC27	S1105	S1240	VDT
1	+	+	+	+	-	+	-	-	-
2	+	+	+	+	+	+	-	-	-
3	+	+	+	+	-	+	-	-	-
4	-	-	-	-	-	-	-	-	+
5	-	-	-	-	-	-	-	-	+
6	+	-	-	-	-	-	-	-	+
7	+	+	+	+	-	-	-	-	+
8	+	+	+	+	+	-	-	-	-
9	-	-	-	-	-	-	-	-	+
10	-	-	-	-	-	-	-	-	+
11	-	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-	-
13	+	+	+	+	+	-	-	-	-
14	+	+	+	+	-	+	-	-	-
15	+	+	-	-	-	-	-	-	-

Genotyping of msp2

Of totally 15 samples tested for the *msp2* gene, 3 (20%) PCR-positive samples had 3D7 alleles and 4 (26.7%) had FC27 alleles (Table 2). In this research FC27 alleles were higher compared to 3D7. There is no information on the polyclonal or monoclonal infected *P. falciparum* isolates. The positive results of the *msp2* gene are marked by the appearance of the band on the PCR amplification results (Figure 3). Even though there is no direct relationship between *msp-2* and SP resistance, *msp-2* genotyping is very important as it can be used to differentiate recrudescence, as well as marker to investigate the genetic diversity, multiplicity of infection, the level of malaria transmission, and immunity against malaria [23], [24], [25].

Various drug resistance analysis has been done mainly in east part of Indonesia as the most malarious provinces [8],[19],[26]. Here a very limited number of samples from Papua were analyzed their resistance to SP based on *dhps* and *dhfr* gene mutations. In this study the number of samples that undergo polymorphism-based mutations is quite high. But these results are still lower than the results of other studies that found *Pfdhfr* mutations up to 100% each in Tanzania [27], in the Amazon [28], in Iran [29] and in East Timor [30]. At different locations de Almeida [30] found mutations of up to 97% (62 of 64). A high percentage of mutation was also found at 95% in Mozambique [31], and was quite high at 57% (12/21) in Burkina Faso [32]. For Indonesia areas (Alor and Lampung) these resistances were 71.2% and 87.2%, respectively [33]. This difference is due to differences in detection methods used, alleles or polymorphisms tested and other factors. Output epidemiological studies state that malaria infection will differ between ecotypes or ecology and the world's climate, even between small ecological sectors, so malaria is a local or focal disease [34].

In malaria endemic regions such as in Papua, *P. falciparum* infection is characterized by extensive genetic diversity which is important information about the local malaria situation. Here in this research *msp-2* gene that is associated with an immune response was analyzed its polymorphisms. The genetic diversity of *P. falciparum* has been extensively studied in many parts of the world [6], [7], [9], [19], [20], [27], [28], [29], [30], but quite limited data are available from Indonesia especially in Papua. Therefore, this study was conducted to understanding that the genetic diversity of malaria parasites in residual transmission foci can provide invaluable additional information in the intervention strategies used to reach elimination targets such as malaria vaccine treatment.

The research implications are that there is need to conduct further high powered study to explore alternative avenues such as vaccine if it is to be beneficial. Beside that this genotyping in SP drug resistance, even though this drug is not used

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for *P. vivax* treatment in Indonesia [26], is important in order to improve the national antimalarial drug policy. Although the existing conventional vector control interventions are competent to minimize the malaria burden considerably, they remain unproductive to eradicate malaria. In this context, introduction of malaria vaccine could be one of the most sustainable and cost-effective approach in the malaria prevention and control strategy [16]. Preliminary study revealed that the successful use of irradiated plasmodium as a vaccine materials depends on finding the radiation dose and dose rate which significantly reduces the pathogenic effect of the microorganism without seriously impairing their immunogenic power [18].

PCR-based mutation detection techniques such as RFLP are an alternative technique that are useful for studying genetic variation in a population [13], [35]. This enzymatic chain reaction is a molecular biology technique that enables the enzymatic synthesis process to amplify DNA fragments [36]. PCR techniques are now commonly used especially in the fields of molecular biology and biomedicine. PCR is the most widely used technique in molecular biology because the process is fast and simple [37].

CONCLUSION

Of the 15 samples tested for *P. falciparum*, it was found that for *dhfr* and *dhps* genes, there were 9 and 8 positive samples mutated in both alleles based on RFLP, respectively. For *P. vivax*, it was found that for *dhfr* and *dhps* genes, there were 0 and 6 positive samples mutated in both alleles based on RFLP. It was concluded that mutations causing parasitic resistance to SP were quite high as was evident in this study that alarming the need for developing vaccine.

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